EXPRESSION OF B7-H3 IN HEALTHY AND DISEASED GINGIVAL SAMPLES – AN IMMUNOHISTOCHEMICAL STUDY

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BRANCH II

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CERTIFICATE

This is to certify that this dissertation titled "**Expression of B7-H3 in Healthy and Diseased Gingival Samples – An Immunohistochemical Study**" is a bonafide record of work done by **Dr. Saumya John** under my guidance during the study period of 2010-2013.

This dissertation is submitted to **THE TAMIL NADU Dr. MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVIATIONS

1.	APC	-	Antigen Presenting Cell
2.	APES	-	3 amino- propyl tri-ethoxysilane
3.	BCR	-	B Cell Receptor
4.	BOP	-	Bleeding on Probing
5.	CAL	-	Clinical Attachment Loss
6.	CD	-	Cluster of Differentiation
7.	CD40L	-	CD40 Ligand
8.	CRS	-	Chronic Rhinosinusitis
9.	CTLA-4	-	Cytotoxic T-Lymphocyte Antigen -4
10.	DAB	-	diamino benzidine
11.	DC	-	Dendritic cell
12.	DPX	-	di-n- butyl phthalate in Xylene
13.	EDTA	-	ethylene diamino tetraacetic acid
14.	HSS-HRP	-	High Sensitivity Streptavidin conjugated to Horse Radish Peroxidase
15.	ICOS	-	inducible costimulatory
16.	IFN-γ	-	Interferon – gamma
17.	IL	-	Interleukin
18.	LI	-	Labeling Index
19.	MHC	-	Major Histocompatibility Complex

20.	NF-κB	-	Nuclear Factor Kappa B
21.	PBS	-	phosphate buffered saline
22.	PD	-	Probing Depth
23.	PD-L	-	Programmed Cell Death-1 Ligand -1
24.	PD-L2	-	Programmed Cell Death -1 Ligand -2
25.	RCC	-	Renal Cell Carcinoma
26.	TCR	-	T Cell Receptor
27.	Th-1	-	Type 1 helper T cell
28.	Th-2	-	Type 2 helper T cell
29.	TREML-2 / TLT-2	-	Triggering Receptor Expressed on Myeloid Cell (TREM) Like Transcript 2

ABSTRACT

Background

T cells have been shown to play a role in etiopathogenesis of periodontal disease. B7-H3, a costimulatory molecule is found to be associated with regulation of T cell function in other chronic inflammatory diseases.

Materials & methods

Gingival samples were taken from 2 groups of patients (group A - periodontal health and group B- periodontal disease). These were paraffinised and processed to carry out immunostaining to identify B7-H3 expression. The slides were then examined under light microscope to assess the positive staining in epithelium and connective tissue. The intensity of positive staining in epithelium and the number of positive cells in the connective tissue were evaluated. Statistical analysis was done using kappa analysis and independent 't' test.

Results

Kappa analysis revealed good inter-examiner agreement for both the groups (group A - 0.718; group B - 0.797). Intensity of staining in the epithelium ranged from intense to moderate for both the groups. In the connective tissue there was a statistically insignificant decrease

(p value- 0.415) in the number of positive cells from group A (mean labeling index- 50.28±26.09) to group B (mean labeling index- 41.37±25.29).

Conclusion

B7-H3 expression demonstrated a statistically insignificant decrease in disease as compared to health.

Keywords :

B7-H3, Periodontitis, Immunohistochemistry.

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INTRODUCTION

Periodontal disease is multifactorial in nature, where the chronic inflammation is initiated in response to bacteria eventually resulting in destruction of tooth supporting structures. This unpredictable but exacerbated host response has been considered responsible for the tissue destruction seen in periodontitis.[17] T cells have been documented to play a central role in modulating host response against periodontopathogenic bacteria.[119]

T cells have demonstrated greater ability to recognise protein antigen in periodontopathogens when compared to other surface components. T cell activation includes antigen recognition, processing and costimulation. The antigen processing is a function that is carried out by both professional and non-professional antigen presenting cells (APC).

Costimulation is carried out by the interaction of B7 ligands on the APC to its corresponding receptor on the T-cell. This process of costimulation ensures that T cell apoptosis is prevented, thereby ensuring that the entire repertoire of T cell cytokines is released.[41,56,72,22,45] The costimulation that has been identified in periodontal disease belongs to the B7-1 & B7-2 members of B7 family.[46] They carry out costimulation through binding to CD28 and inhibition by binding to CTLA-4.

In recent years the role of B7-H3, a newly identified member of B7 family of costimulatory molecules has been proposed to contribute in the

etiopathogenesis of various non-linear inflammatory diseases. Several studies have reported either an increase or decrease in B7-H3 expression.[23,73,147]

In addition to immune cells, human B7-H3 has been shown to be expressed by non-immune cells.[61,134] Early studies reported that the binding of B7-H3 to its counter-receptor on T cells leads to T cell activation.[23] However other studies have reported an inhibitory role for B7-H3 in T cell activation.[134,135,147]

The clinical course of periodontal disease is characterised by short bursts of disease activity and long periods of quiescence. The factors that have been proposed to play a role are not yet well defined. They are thought to be related to a change in quantity or quality of periodontopathogenic bacteria and/or host response. The host response in turn is thought to be determined by preponderance of either Th1 or Th2 lesion.[127,38] Even so, the factors that upregulate the inflammatory process or T-helper cells are better defined when compared to inhibitory mechanism.

There is as yet limited information about the nature of signals that inhibit or regulate the T-helper cell response in periodontal disease. It has been postulated that B7-H3 might play a regulatory role in T-helper cell response, similar to other chronic inflammatory lesions such as rheumatoid arthritis.

AIMS & OBJECTIVES

The aim of the present study was

- To evaluate if gingiva expresses B7-H3 (immunoregulatory/ costimulatory molecule)
- To compare B7-H3 expression between healthy and diseased gingival tissue samples.

REVIEW OF LITERATURE

Periodontitis is a chronic, non-resolving inflammatory disease of multifactorial etiology characterized by destruction of tooth supporting structures in susceptible individuals.[47] Disease progression is due to a combination of factors such as genetics, environment and the host immune system. These factors may modify the pathway by which infectious agents cause inflammation and therefore can determine whether microbial infection will lead to periodontal destruction or to periodontal stability.[63]

The destruction of bone and connective tissue, including collagens, proteoglycans, and other components of the extracellular matrix does not follow a linear pattern. The tissue destruction is constantly being adjusted by the host-bacterial interactions resulting in periods of exacerbation and quiescence. In diseased periodontal tissues there is a dense mononuclear inflammatory infiltrate between the bacteria and the tissues.[111] This infiltrate contains all of the cellular components necessary to control the infection, including abundant T lymphocytes[43,140] as well as numerous B cells and plasma cells.[128] The central role played by T cells in regulating immune response to periodontal disease have been well-documented.[38,113,127]

GINGIVA

The oral epithelium represents a dynamic physical and chemical barrier against the microbial biofilm.[12] Histologically, healthy gingival tissues

comprise keratinocytes and also immune cells like dendritic cells,[26,36,69,70] polymorphonuclear leukocytes,[125] lymphocytes and mononuclear phagocytes.[125]

Cells of junctional epithelium secrete cytokines including chemotactic cytokines (IL-8[145,146] and cytokine-induced neutrophil chemoattractant-2[101]), as well as proinflammatory cytokines (IL-1 and tumor necrosis factor-α[101]). Furthermore, cells of the junctional epithelium and their resident leukocytes contribute to host defense by expression of antibacterial peptides including calprotectin,[107] α- and β- defensins[149,24,86] and cathelicidin LL-37.[34] **Vankeerberghen A et al** (2005)[149] demonstrated an upregulation of β-defensins and IL-8 by polymorphonuclear leukocytes, in tissues of patients with chronic periodontitis.[86] Studies by **Bissell J** (2004)[10] and **Lundy FT**(2005)[88] stated that healthy tissues appear to have high concentrations of defensins, indirectly supporting their role in homeostasis of the gingival sulcus in health .

Emerging evidence indicates that keratinocyte activation is a result of interaction between periodontal bacteria and keratinocytes, leading to release of a variety of inflammatory mediators.[39] These stimuli may act locally to transmit the inflammatory message in a centripetal direction towards the subepithelial microvessels and thereby induce an inflammatory reaction.[75]

Antigen presenting cells

The microbe must first be recognized and captured in the skin/mucosa by APC. For this they possess a unique array of antigen recognition and capture receptors including the toll-like receptors and C type lectin receptors. The antigen must then be processed into smaller peptides. Following which, the peptides must then be packaged with molecules called major histocompatibility complex (MHC) and presented to the T cells.[129,150]

APCs are further classified as professional APC (dendritic cells, macrophages and B-cells) and non- professional APC (keratinocytes and gingival fibroblasts). The professional APCs express MHC class II molecules and costimulatory molecules.[31,5]

Gingival keratinocytes

Renne J et al (2010)[120] and **Chung Y et al** (2009)[25] reported the constitutive expression of IL-1α on keratinocyte which has been known to promote T cell responses. It has autocrine actions which include upregulating MHC class II expression and, stimulation of cytokine and chemokine production. Besides, it also has juxtacrine actions to activate neutrophils, monocytes and T cells which infiltrate under inflammatory conditions.[132] It has been reported that IL-1α was a general amplifier of T-cell responses in several epithelial tissue immune responses.[18,105,154]

Dendritic cells

They occupy a unique niche in the innate immune system by serving as a bridge to the adaptive immune system. They do this by capturing microbes and their antigens while in the immature state and stimulating a T cell response to these antigens in their mature state. They enable in priming naive helper/cytotoxic T cells to undergo clonal expansion.[5,6,31,57]

To stimulate naive T cells that have never encountered the antigen before, additional signals (i.e. costimulatory signals) which are expressed by dendritic cells are required. Dendritic cells express class II MHC molecules also costimulatory molecules CD80 and CD86.[31,6]

In peripheral tissues of humans, three major types have been described, including two of myeloid origin – Langerhan's cells and interstitial dendritic cells and the third of lymphoid origin- plasmacytoid dendritic cells.[31] Others include follicular dendritic cells (restricted to primary B-cell follicles)[76] and those developed from in vitro dendritic cell culture systems (cultured either directly from CD34+ hematopoietic progenitors[20,21] or precursors differentiated from CD34+ progenitors).[69,143] CD1a+ Langerhan's cells have been found to increase in number in the epithelium with gingivitis,[69] experimental gingivitis[106,151] and periodontitis.[26,69] It is speculated that these Langerhan's cells are mobilized and matured in response to inflammatory

cytokines and pathogen associated molecular patterns from oral mucosal pathogens.[69,30,32]

Macrophages

Circulating monocytes can migrate to any site of infection or inflammation where they transform into macrophages which phagocytose and kill microbes. Macrophages constitutively express low levels of MHC class II molecules and costimulators. In the presence of effector Th1 cells which secrete higher levels of IFN-γ and CD40L macrophages carry out antigen presentation. CD40-CD40L binding ensures that macrophages present antigen to T cells.[1]

B-cells

Its antigen presentation differs from other profesional APC (dendritic cells, macrophages). They engulf antigen using immunoglobulin receptor referred to as the B cell receptor. The antigen is reduced to peptides and transported to B cell surface for presentation to CD4+ T cells. For clonal expansion and costimulation apart from interaction of BCR and antigen, binding of CD40 (B cell) to CD154 (activated T cell) is essential.[8]

Gingival fibroblasts

Fibroblasts can modulate immune cell behaviour by conditioning the local cellular and cytokine production. Fibroblast activation leads to

production of cytokines, chemokines and prostanoids such as prostaglandin E2.[133] Fibroblasts can also regulate the behaviour of haematopoietic cells present in damaged tissue via CD40–CD40L interaction, which leads to the activation of the NF-kB family of receptors. This causes fibroblasts to synthesize high levels of IL-6, IL-8, cyclooxygenase-2 and hyaluronan.[166,112] This mechanism is similar to the crosstalk that occurs between lymphocytes and antigen presenting cells and suggests that it may provide crosstalk between fibroblasts and leukocytes. **Brouty-Boye D et al** *(*2000)[15], **Hogaboam C.M. et al** (1998)[62] and **Pap T et al** (2000)[112] reported that fibroblasts taken from diseased tissues display a fundamentally different phenotype compared with fibroblasts taken from normal tissues at the same anatomical site.

ROLE OF T CELLS IN PERIODONTAL DISEASE

T cells are involved in nearly all immunoregulatory interactions both in vivo and in vitro[118] and a delicate balance between effector and regulatory subsets is required for immune homeostasis.[119]

T helper cell subsets

Mosmann et al (1986)[103] first reported that mouse T cell clones can be classified as two functional subsets based on their cytokine profile as type I helper T cells (Th1) and type 2 helper T cells (Th2). The 2 distinct CD4+ helper T-cell subsets are induced following infection whose balance

determines what kind of adaptive immune response is utilized to eliminate infection.[103] Th1 cells preferentially secrete interleukin-2 (IL-2), interferon- γ (IFN-γ)[103] and result in cell mediated immunity.[27] They increase the ability of macrophages to kill both intracellular and extracellular pathogens and also mediate delayed-type hypersensitivity reactions.[121] Th2 cells secrete IL-4,5,6 and 13[103] and induce humoral immunity resulting in B-cell development.[104] **D'Andrea A et al** (1993),[33] **Ohmori et al** (1997)[109] showed that once either Th1 or Th2 cells become dominant they can dampen the development of the other subset, as a result making it difficult to change the immune response pattern.

Factors influencing Th subsets

Differentiation of Th1 and Th2 T-cell subsets is determined during priming and is influenced by a number of factors, including the cytokine environment, the antigen itself, antigen dose, the route of administration, the nature of the antigen presenting cells and costimulatory molecules.

1. Antigen binding

The huge spectrum of antigen diversity of T cells is achieved by structural variation of TCR. TCRs consist of two polypeptide chains, α and β or γ and δ chains. Diversity in antigen recognition by T cells is generated in part by the recombination of germ line V, D, J and C gene segments of TCR β and δ chain and V, J and C of TCR α and γ chain.[35] **Boyton RJ** (2002)[13]

studied the impact of TCR selection and structure in Th1 and Th2 lines and clones with fixed peptide specificity and class II restriction. The Th2 clones tended to use TCR complimentarity–determining region (CDR) 3α loops than their Th1 counterparts. Molecular modeling of Th1- and Th2 – derived TCR showed that Th2 CD3α comprised larger side chain residues than Th1 TCR. It was proposed that under Th2 polarizing conditions, there was a trend for CD4+ T cells to have elongated TCR CD3α loops, which are predicted to alter TCR binding and reduce contact at other interfaces, possibly impeding TCR triggering. **Boyton et al** (2002)[13] concluded that either the elongated receptor was lost during selective expansion of Th1 cells or that selection of the Th2 line was compatible with expansion of cells bearing either type of receptor, with the elongated form as the preferred receptor.

2. Cytokines

Boyton & Altmann (2002)[14] have proposed that differential selection from the available pool of specific TCR occurs during Th1 or Th2 development. They also suggested that programming to select for cells to become either Th1 or Th2 T cells may come from local factors such as cytokine or chemokine milieu. They also stated that while many factors determine the polarization of T cells any one factor can override the changes initiated by any other factor.[14]

Iezzi G (1999)[65] reported that in the presence of IL-12, a short T cell receptor stimulation has been shown to induce Th1 polarization, IL-12

exerting its effect during and after TCR signaling. Th2 polarization, on the other hand required prolonged TCR signaling and IL-4 was effective only when present during TCR triggering. These authors concluded that duration of TCR stimulation was crucial in influencing Th1/Th2 polarization.

3. Antigen presentation

The regulation of the immune response may reside at the level of antigen presentation such that presentation by professional antigen-presenting cells (such as dendritic cells and macrophages) results in stimulation of destructive cytokines while presentation by nonprofessional antigen-presenting cells (such as epithelial or endothelial cells) results in anergy and no tissue destruction.[59,142] The mechanism of this anergy is unknown, but it is well established that junctional and pocket epithelial cells express major histocompatibility complex class II antigens[127] and hence could be capable of antigen presentation, which results in anergy and possible apoptosis of the Thl cells, and thereby allow cells with a Th2 cytokine profile to emerge. Endothelial cells, which in humans constitutively express major histocompatibility complex class II, could also present antigen leading to anergy.[142]

Where B7 ligands are limited, low levels of CD152 with its greater affinity and avidity for B7 may induce signals which predominate and inhibit T cell responses. With increased expression of B7, initially on dendritic cells

and subsequently on activated B cells, CD28 signals may predominate, resulting in T cell activation.[22]

T cells express CD28 rather than CD152 in periodontal disease specimens.[46,74] CD28+ T cells were found to increase with increasing numbers of B cells suggesting T and B cell interactions with increasing inflammation.[46]

CD28/B7 interactions may influence cytokine profiles, with Th1 clones being more dependent on B7 than Th2 clones.[45,66] Th2 clones on the other hand which use IL-4 as their autologous growth factor, can be activated without CD28 costimulation,[99] indicating that activation of differentiated Th2 cells may require other costimulatory molecules.[45] However B7/CD28 binding is necessary to make these cells responsive to IL-4. CD28 increases responsiveness to IL-4 through an IL-1 dependent route, i.e., CD28 costimulation is mediated by IL-1 induction in these cells. Therefore, CD28 is required for activation and proliferation of Th1 and Th2 cells although the mechanism may differ.[99]

Th1/Th2 paradigm

Seymour et al (1993)[127] proposed that in non-susceptible individuals predominantly Th1 subsets are found leading to cell mediated immunity. IFN-γ expression is upregulated and protective antibodies maybe produced. However in individuals susceptible to periodontal breakdown Th2 subsets

predominate. They produce cytokines required for B cell proliferation and differentiation leading to polyclonal B cell activation and upregulation of non-protective antibodies along with IL-1 production by B cell.

Ebersole and Taubman (1994)[38] carried out adoptive transfer experiments using the Th2 clone A3. They reported that Th2 cells abrogate periodontal disease and Th1 cells aid in disease progression.

Pilon M et al (1991)[113] demonstrated decreased Th1 cytokines in the gingival crevicular fluid of periodontitis sites. **Fujihashi K et al** (1991)[42] reported decreased Th1 cytokines in gingival mononuclear cells of periodontitis sites. **Sigush B et al** (1998)[131] and **Gemmell E** (1994)[48] reported decreased Th1 cytokines in peripheral blood mononuclear cells from periodontitis patients stimulated with mitogens, P.gingivalis and F.nucleatum respectively. **Aoyagi T et al (1995)**[2], **Bartova J et al (2000)**[7], **Yamazaki K et al** (1994)[158] reported increased Th2 responses in peripheral blood from patients with periodontitis . **Lappin DF et al** (2001)[78], **Tokoro Y et al** (1997) and **Yamazaki K et al** (1994)[158] reported increased Th2 responses in gingival tissues. **Manhart SS et al** (1994)[95] and **Reinhardt RA et al** (1989)[117] reported increased Th2 responses in extracted gingival mononuclear cells and gingival crevicular fluid respectively. All these studies support the hypothesis that Th1 cells are associated with a stable lesion and a Th2 response with disease progression. However, **Ebersole JL et al** (1994),[38] **Salvi GE** (1998),[124]

Takeichi O (2000)[140] reported a predominance of Th1-type cells or reduced Th2 responses in periodontally diseased tissues.

Fujihashi K et al (1994)[44] and **Prabhu A et al** (1996)[115] have suggested the involvement of both Th1 and Th2 cells in sites with periodontitis. mRNA for both Th1 and Th2 cytokines has been demonstrated by these studies in periodontally diseased tissues.

Activation of T cells

Antigen recognition is the first step in the clonal expansion and activation of antigen-specific T cells. They recognize peptide antigens via the TCR. On the surface, TCR is expressed as a complex with a CD3 molecule whose ζ chains have cytoplasmic tails capable of transmitting an activation signal derived from antigen binding to TCR.[94] CD4 and CD8 molecules act as coreceptors with TCR.[9] For T cell to recognize an antigen, the antigen has to be engulfed by APC, processed into antigen peptides and presented on the cell surface by binding to peptide binding groove of MHC. The TCR recognizes and binds to the antigen bound MHC presented by APC.[129,150] This provides the 1st signal for T cell activation leading to proliferation, differentiation and activation of CD28. The second signal is non-specific resulting from binding of APC expressed costimulatory ligands (belonging to B7 family) to its receptor on T cell which is important for T cell survival and function. However T cells cannot be activated by only an antigen signal. Without

costimulatory signals from costimulatory molecules such as CD28[68,71] and the CD40L,[3] antigen signals make T cells anergic rather than activate. It is now accepted that optimal activation of T cells requires both costimulation and TCR engagement. [41,56,72,22,45]

SECONDARY SIGNALING MOLECULES

Costimulatory interactions between the B7 family ligands expressed on antigen-presenting cells (APC) and their receptors on T cells play critical roles in the growth, differentiation, and death of T cells.[41,56,72,22,45]

CD28 & CTLA4

A costimulatory signal (signal 2) occurs through the CD28 molecule, which is recruited to the immunological synapse following TCR ligation and is provided by B7-1 or B7-2. Like the MHC, the B7 proteins are expressed by APCs. The costimulatory signal serves to induce T-cell production of interleukin (IL) -2. IL-2 acts in an autocrine/paracrine fashion on the T cells and is obligatory for their survival and differentiation into effector cells.[114] Without the costimulatory signal, signal 1 from the TCR by itself induces T cells to become tolerant to their cognate antigen instead of activated.[67,50,51] Both the TCR and CD28 are constitutively expressed on most naıve T cells, such that the T cell is ready to respond to antigen as presented by an MHC-expressing APC.

Cytotoxic T lymphocyte antigen 4 (CTLA-4) functions to inhibit T-cell responses and thus has opposing activities to CD28. Binding of B7-1 or B7-2 with CTLA-4, a homolog of CD28, may inhibit T cell responses by delivering a putative negative signal.[22,16,77,152,153]

B7 Molecules

B7 family comprises of immunomodulatory proteins required for fine-tuning immune responses in addition to the primary signal provided by peptide-MHC complex. Several authors have reported that costimulatory interactions between the B7 family ligands expressed on antigen-presenting cells (APC) and their receptors on T cells are essential for the growth, differentiation, and death of T cells.[41,56,72,22,45]

These mediate cell-to-cell interaction by ligating surface associated receptors belonging to the CD28 family, expressed by T- lymphocytes. Members of the B7 family include – B7-1, B7-2, ICOS-L, B7-H1 (PD-L1), B7-DC(PD-L2), B7-H3 and B7-H4. They belong to the immunoglobulin (Ig) superfamily of type I transmembrane proteins.

B7-1 & B7-2

B7-1 (CD80) and B7-2 (CD86) are the best-described B7 proteins. They interact with the co-stimulatory receptor CD28 and the inhibitory receptor CTLA-4.[71,54] B7-1 and B7-2 are expressed mainly by professional

antigen presenting cells which are activated monocytes, B cells and dendritic cells.[71,54]

Macrophages constitutively express low levels of CD86. CD80 is induced after treatment with interferon-γ. CD86 expression is also low on B cells until activation, which induces a rapid up-regulation of these molecules.[66] **Gemmell E et al** (2001)[46] reported that the percentage of CD86+ B cells and macrophages has been shown to be significantly higher than the percentage of CD80+ macrophages in gingival tissue sections. **Mahanonda R et al** (2002)[92] showed that CD86 was up-regulated mostly on B cells isolated from periodontitis lesions. They also reported that a number of periodontopathogenic bacteria including P. gingivalis up-regulated CD86 on B cells in vitro.

Azuma M et al (1993)[4] has reported the expression of B7 molecules by activated human peripheral blood T cells, CD4 and CD8 clones and natural killer clones. **Hirokawa et al (1995)**[60] suggested that B7 molecules functioned as co-stimulatory molecules on T cells and helped in clonal expansion of activated T cells. Another study has shown that memory CD4 cells express CD86 while naive CD4 cells do not. Naive T cells expressed CD80 after co-stimulation with CD86 and T cell receptor ligation.[55]

B7-H1 (PD-L1) & B7-DC (PD-L2)

B7-H1[37] and B7-DC[148] (also referred to as PDL-1 and PDL-2), are ligands for PD-1, an inhibitory receptor on T cells.[79] B7-H1 (PD-L1) and B7-DC (PD-L2) have been identified in both lymphoid and several nonlymphoid tissues, as well as in several tumor cell lines, and are putative inhibitory co-stimulatory ligands.[79] Interaction of these ligands with the counter-receptor PD-1 can result in inhibition of T and B cell responses.[108] PD-1 receptor is thought to mediate immunological self-tolerance. B7-DC can inhibit lymphocyte activation, or, like B7-H1, co-stimulate lymphocyte function through an as yet ill-understood mechanism.[148,79] However, several authors have found that binding of PD-L1 and 2 to PD-1 is costimulatory on T cells.[37,148,130,141,84]

B7-H2 & ICOS

B7-H2 is detected on B cells and macrophages, and is a ligand for the inducible costimulatory (ICOS) expressed on antigen-primed T cells. Costimulatory signals via ICOS activate memory T cells with some preference for Th2 responses.[155,28,97,98,64] **Yoshinaga SK et al** (1999)[160] reported its expression by activated and resting memory T cells. Several authors have reported that it is constitutively expressed by B lymphocytes and is induced by tumor necrosis factor α on nonlymphoid cells.[82,155,160,139] **Chapoval AI et al** (2001)[23] has shown that engagement of B7-H2 results in activation of T-helper

memory cells with a bias production of Th2 cytokines, such as IL-4 and IL-13.[81]

B7-H3

Chapoval AI et al (2001)[23] discovered B7-H3, a B7 homologue, in the laboratory of Lieping Chen. They originally described B7-H3 as a 2-Immunoglobulin like domain. This was supported in murine studies done by **Sun M et al** (2002)[136] and **Ling V et al** (2003).[83] Mouse B7-H3 is located on chromosome 9, which shares 88% identity and 93% similarity with the human molecule. Human B7-H3 is encoded on chromosome 15. **Steinberger P et al** (2004)[134] reported that the human B7-H3 has a 4 Ig-like structure. They proposed that the 4 Ig structure could be due to a duplication of the locus, which encodes B7-H3-IgV and -IgC exons.

Chapoval AI et al (2001),[23] **Sun M et al** (2002),[136] **Zhang GB et al** (2005)[165] and **Steinberger P et al** (2004)[134] have reported its expression is induced in T cells, B cells, monocytes, dendritic cells (DCs) and some tumour cell lines. B7-H3 is also found on non-professional APCs including fibroblasts, fibroblast-like synoviocytes, and epithelial cells.[61]

At the transcriptional level, B7-H3 is found in most organs.[23,136,165] At the protein level, B7-H3 is found in human liver, lung, bladder, testis, prostate, breast, placenta, and lymphoid organs. **Hofmeyer KA et al** (2008)[61] suggested

that due to its posttranscriptional regulation different expression patterns have been observed between B7-H3 mRNA and protein.

Zhang G et al (2008)[163] demonstrated that human soluble B7H3 (sB7-H3) was cleaved by MMPs from the surface of activated T cells, monocytes and monocytes-derived dendritic cells which was able to bind its receptor on activated T cells.

Zhang G et al (2010)[164] reported that sB7-H3 on binding to costimulatory receptors on monocytes /macrophages results in secretion of proinflammatory cytokines.

Zang X et al (2003)[161] and **Hashiguichi et al** (2008)[58] reported that triggering receptor expressed on myeloid cell (TREM)-like transcript 2 (TLT-2, or TREML2), is the costimulatory receptor for B7H3 particularly in CD8 T cells. However, **Leitner et al** (2009)[80] showed that B7-H3 is not a costimulatory ligand for TREML-2 receptor. Also several authors have reported that B7-H3 is an inhibitory molecule. This function has been reported to be mediated by its binding to another receptor.[61]

Cell culture studies

B7-H3 coinhibitor

Mahnke et al (2007)[93] reported that on contact between dendritic cells (DC) and CD4+CD25+regulatory T cells (Tregs) there was an upregulation of

B7-H3 molecule on DC surface. Also the number of MHC-peptide complexes were decreased.

Steinberger P et al (2004)[134] reported B7H3 is expressed by immature and mature monocyte derived dendritic cells. They found that it binds to an inhibitory receptor on T cells as its binding does not stimulate T cell proliferation.

Murine studies

B7-H3 costimulator

Chapoval AI (2001)[23] reported B7-H3 Ig fusion protein increase CD4+ and CD8+ T cells and selectively stimulates IFN-γ production.

Hashiguichi M et al (2008)[58] reported that binding of B7-H3 to TLT-2 results in proliferation of CD8+ T cells and upregulation of IFN-γ production by CD8+ T cells.

B7-H3 coinhibitor

Prasad DV et al (2004)[116] reported that it is expressed by all professional APCs and a minor subset of CD4+ and CD8+ T cells. It was found to inhibit transcriptional factors AP-1, NFAT and NF-κB which regulate T cell activities. Murine B7-H3 was found to inhibit T-cell activation and effector cytokine production.

Human studies

B7-H3 coinhibitor

Ling V et al (2003)[83] reported that B7-H3 downregulates CD4+ T cell proliferation and effector cytokine production.

B7-H3 EXPRESSION IN SYSTEMIC DISEASES

Animal studies

B7-H3 coinhibitor

Suh WK et al (2003)[135] detected that B7-H3 deficient mice developed more severe airway inflammation and the lesions had predominantly Th1 cells. They reported that B7-H3 is upregulated in the presence of IFN-γ and downregulated in the presence of IL-4.

Prasad DV et al (2004)[116] found that when B7-H3 deficient mice were exposed to autoimmune encephalomyelitis, it was exacerbated.

Human studies

B7-H3 costimulator

Kim J et al (2005)[73] found that B7-H3 was the most abundant costimulatory molecule detected by flow cytometry on cultured respiratory tract epithelial cells. Engagement of B7-H3 ligands results in proliferation of CD4+ and CD8+T cells, a bias toward Th1 cytokine production, and primary

cytotoxic T cell activation. They concluded that the presence of B7 homologs on epithelial cells may play a role in driving expression of the Th1 and Th2 cytokines observed in asthma and CRS.

B7-H3 coinhibitor

Tran et al (2008)[147] found that B7-H3 expression was associated with FLS rich areas and was in close proximity to T cells in the RA pannus. The expression of B7-H3 was found to be constitutive and uninfluenced by immunoregulatory cytokines. They suggested that B7-H3 could be an important signaling molecule between FLS and T cells. They reported that B7-H3 binds to inhibitory receptor on unstimulated T cells and activating receptor on cytotoxic T cells.

Cancer immunity

In vitro

B7-H3 costimulator

Studies have been carried out with commonly used human cancer lines in the laboratory, such as HL-60 promyelocytic leukemia, K562 myelogenous leukemia, SW480 colon adenocarcinoma, A549 epithelial lung adenocarcinoma, and G361 melanoma. They were found to express high level of B7-H3 mRNA.[23]

Animal Studies

B7-H3 coinhibitor

Sun X et al (2003),[137] **Luo L et al** (2004),[90] **Luo L et al** (2006)[89] and **Lupu CM et al** (2006)[91] reported that in mouse cancer models, ectopic expression of B7H3 leads to activation of tumor-specific CTLs that are able to slow, tumor growth or even completely eradicate tumors.

Human studies

B7-H3 costimulator

Many human cancers have been found to naturally express B7-H3. Subsequent studies demonstrate that prostate cancer, non-small-cell lung cancer, gastric carcinoma, ovarian cancer, renal cell carcinoma, urothelial cell carcinoma, and neuroblastoma, also express B7-H3.[19,23,29,138,162,156,123,11]

Crispen PL et al (2008)[29] showed that B7-H3 expression by either clear cell renal cell carcinoma (RCC) or the tumor vasculature was found to significantly associate with an increased risk of death from RCC. Likewise, a marked increase in B7-H3 expression was observed in majority of prostrate cancers, and statistically correlated with poor prognosis.[122] Also in case of urothelial cell carcinoma,[11] non small-cell lung cancer,[157] and pancreatic cancer immunohistological studies show that increase in B7-H3 expression correlates with cancer progression. **Castriconi R et al** (2004)[19] reported that

4Ig B7-H3 protects tumor cells in bone marrow (especially neuroblastoma) from natural killer cell (NK cells) mediated killing. They reported that binding of 4IgB7-H3 to its receptor on NK cells results in inhibition of its actions.

B7-H3 coinhibitor

Studies in gastric carcinoma correlates elevated B7-H3 expression by cancer with an increased survival rate in patients.[162,156] **Loos et al** (2009)[85] examined B7-H3 expression by immunohistochemistry in pancreatic cancer tissue specimens from 68 patients who underwent surgical resection. They found that abundant expression of B7-H3 by tumor cells in pancreatic cancer was associated with increased expression of cytotoxic T cells, and correlated with anti-tumor immune response.

MATERIALS AND METHODS

STUDY POPULATION

The study population comprised of randomly selected patients from those attending the Dept. of Periodontics, Ragas Dental College and Hospital, Chennai. The study protocol was approved by the Institutional Review Board of Ragas Dental College. Prior to conducting the study, informed consent was obtained after explaining the study protocol to the patients. A total of 30 patients were randomly selected for the study.

The selected patients were divided into two groups:

Group A: 15 periodontally healthy subjects (5 males & 10 females of age 20-50 years (mean 35.6 years)) exhibiting no signs of periodontal disease as determined by the absence of bleeding on probing (BOP) and clinical attachment loss (CAL) , with probing depth (PD) $\leq$ 3mm.

Group B: 15 subjects with chronic periodontitis (7 males & 8 females of age 20-65 years (mean 45.7 years)) exhibiting PD $\geq$5 mm, CAL $\geq$ 3 mm and presence of BOP in at least six sites.

Inclusion criteria:

1. Patients who were systemically healthy.

2. Patients who were non-smokers.

3. No history of anti-microbial therapy for the past three months.

Exclusion criteria:

1. Patients who had received periodontal treatment within the previous 6 months.

2. Patients with other dental problems such as pulpal diseases.

GINGIVAL TISSUE SAMPLE COLLECTION

Gingival tissue samples were obtained from periodontally healthy patients (Group A) during crown lengthening procedure, by placing an external bevel incision 1mm from gingival margin (1*3 mm tissue).

In patients with chronic periodontitis (Group B) gingival biopsy was obtained, one week after scaling and root planning (SRP). An internal bevel incision was placed 1mm from gingival margin (1*3 mm tissue) and tissue was excised during the modified Widman flap procedure.

The obtained gingival tissue specimens were fixed with 10% neutral buffered formalin. Prompt fixation was carried out to ensure the preservation of tissue architecture and cell morphology. The tissues were paraffin embedded within 48 hours, to avoid degradation of the antigens.

ARMAMENTARIUM FOR IMMUNOHISTOCHEMISTRY PROCEDURE

Instruments/Equipments

1. Aluminium foil
2. Microscopic slides
3. Detergent solution (Laxbro)
4. Autoclave
5. Beakers
6. Coplin jars
7. Cover slips
8. Electronic timer
9. Slide warming table
10. Light microscope
11. Measuring jar
12. Micropipettes
13. Rectangular steel trays with glass rods
14. Refrigerator
15. Sterile gauze
16. Tooth forceps
17. Diamond pencil
18. Weighing machine (Sartorius)

19. Coulter's chamber

Reagents:

1. Distilled water
2. Acetone
3. Xylene
4. Absolute alcohol
5. Alcohol 70%
6. Alcohol 50%
7. Hydrogen peroxide 3%
8. EDTA buffer (pH8.0)
9. Phosphate buffered saline (pH 7.0)
10. Harris's Hematoxylin
11. DPX

ANTIBODIES:

Primary Antibody:

- R&D systems, Human B7-H3 Affinity Purified Polyclonal Antibody (100UG); Catalog Number AF 1027, Minneapolis , USA.

Immunohistochemistry Kit:

R & D systems Anti-Goat HRP-DAB Cell & Tissue Staining Kit; Catalog Number: CTS008; Minneapolis, USA.

- **Peroxidase Blocking Reagent** - 6 mL of 3% Hydrogen Peroxide (H_2O_2).
- **Avidin Blocking Reagent** - 6 mL of Avidin solution, containing 0.1% Sodium Azide (NaN_3).
- **Biotin Blocking Reagent** - 6 mL of Biotin solution, containing 0.1% NaN_3.
- **"Vial A" Secondary Biotinylated Antibodies** - 6 mL of either anti-mouse, anti-rabbit, anti-goat, anti-rat, or anti-sheep secondary antibodies, respectively, in 0.01 M PBS containing 0.1% NaN_3.
- **"Vial B" High Sensitivity Streptavidin-HRP Conjugate (HSS-HRP)** - 6 mL of Streptavidin conjugated to HRP in 0.01 M PBS containing 1% carrier protein, with preservatives and stabilizer.
- **DAB Chromogen** - 2 mL of 2.5% 3,3-diamino benzidine (DAB) in stabilizing buffer.
- **DAB Chromogen Buffer** - 2 vials (15 mL/vial) of 0.1% H_2O_2 in Tris HCl Buffer.

SLIDE PREPARATION

Precoating procedure

Before taking the sections onto the slide, all the slides were coated with APES (*3 amino- propyl tri- ethoxysilane*). Pre coating procedure of the slides was as follows:

- Slides were first washed in tap water for few minutes.
- They were then soaked in detergent solution (Laxbro) for an hour.
- After 1 hour each slide was brushed individually using the detergent solution and was transferred to distilled water.
- Slides were washed in two changes of distilled water.
- Later slides were washed in autoclaved distilled water.
- The slides were then immersed in 1 N HCl overnight.
- The following day the slides were washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminum foil and baked in hot air oven for 4 hours at 180^{0}C.

APES coating procedure:

The slides were allowed to cool down and were then coated with APES using the following procedure.

- Slides were dipped in a Coplin jar containing acetone for 2 minutes.

- The slides were then dipped in a Coplin jar containing APES for 5 minutes.
- Following this, the slides were dipped in two changes of distilled water for 2 minutes each to remove excess APES and were left to dry.

TISSUE SECTIONING

- The paraffin embedded tissues were sectioned approximately 4 microns thick using a manual rotary microtome.
- The sections were placed in a water bath containing deionized distilled water preheated to 40-44ºC to remove micro-wrinkles.
- Two tissue sections were placed flat, wrinkle and fold free on the APES pretreated microscope slides.
- Tissue sections were dried by placing on a slide warming table at 100^{0}C for 60 minutes.

IMMUNOHISTOCHEMISTRY (IHC) PROCEDURE

Positive control

Prostrate cancer tissue was used as positive control.

Negative control

Gingival tissue to which primary antibody was not added, was used as negative control.

Immunohistochemistry procedure was carried out using R&D systems IHC protocol.

Deparaffinisation of tissues sections

The slides with tissue sections were heated on a slide warming table at 100˚C for 2 minutes. The slides were treated with two changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with Phosphate Buffered Saline (PBS). Circles were drawn around the tissues, so that the antibodies added later did not spread and were restricted to the circle.

Antigen Retrieval

The slides were then transferred in a coplin jar containing EDTA buffer and antigen retrieval was done in an autoclave at a temperature of 121°C and pressure of 20 psi for 15 minutes. Then the slides were allowed to cool till it reached room temperature.

Staining procedure

The slides were treated with 3% hydrogen peroxide for 15 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non – specific staining. The slides were put in one change of PBS. Slides were then treated with avidin blocking agent (R&D systems, CTS008, Minneapolis,

USA) for 15 minutes. Slides were rinsed in PBS following which the slides were treated with biotin blocking agent for 15 minutes. Avidin and biotin blocking agents were used to suppress endogenous avidin binding activity (EABA). Slides were again rinsed in PBS.

The primary antibody B7-H3 (R&D systems, AF1027, Minneapolis, USA) was added to the tissue on the slides. The stainless steel tray with glass rods containing the slides was incubated overnight at 2-8℃. The slides taken out were washed in three changes of cold PBS for 5 minutes each, to remove the excess antibody. Then the slides were wiped carefully without touching the tissue section to remove excess PBS. Then 1-3 drops of biotinylated secondary antibody (R&D systems, CTS008, Minneapolis, USA) was added onto the sections and the slides were incubated for 1 hour. Later the slides were washed in three changes of cold PBS for 15 minutes each. The slides were again wiped carefully without touching the tissue section to remove excess PBS. Then 1-3 drops of HSS- HRP from the secondary antibody kit (R&D systems, CTS008, Minneapolis, USA) was added onto the sections and the slides were incubated for 30 minutes. The sections were then washed in three changes of cold PBS for 2 minutes each. Then the slides were again wiped carefully to remove excess PBS. Then a drop of freshly prepared DAB (diamino benzidine – a substrate Chromogen) (R&D systems, CTS008, Minneapolis, USA) was added onto the sections and incubated for 15 minutes. Slides were then

washed in three changes of PBS for 10 minutes each and then rinsed in distilled water to remove excess DAB and counter stained with Harris's Haematoxylin. They were then washed with alcohol and xylene. The tissue sections were mounted with DPX. Throughout the procedure, care was taken not to dry the tissues.

Observation:

All the slides were viewed under the light microscope at magnification 10x and 40x. The slides were checked for positive staining in the tissue sections. The strong brown membranous immunostaining exhibited by the gingival tissue was taken to be positive. Thus the intensity of staining (in epithelium) and the number of cells (counted in the connective tissue) was recorded.

Positively stained cells were seen in the basal layers of oral gingival epithelium in all the slides examined. The intensity of staining was observed by two examiners and graded as Negative (-), Mild (+), Moderate (++) and Intense (+++). The inter-examiner variation was determined by kappa analysis.

In the connective tissue positive stain was uniformly identified in the cells present just beneath the basement membrane. The number of positive

cells in the connective tissue was manually counted using Coulter's chamber and the mean labeling index was calculated.

$$LI = \frac{\text{Number of positive cells}}{1000} \times 100$$

IHC PROCEDURE FLOW CHART

APES coated slides with sections

preheated on slide warming table (100^0C;2mins)

↓

Placed in xylene I (10 min)

↓

Placed in xylene II (10 min)

↓

Placed in 100% isopropanol (10 min)

↓

Placed in 70% isopropanol (5 min)

↓

Placed in 50% isopropanol (5 min)

↓

Washed in PBS (10 mins)

↓

Kept in EDTA buffer and autoclaved (20 psi;15 mins)

↓

Cooled to room temperature

↓

Placed in 3% hydrogen peroxide (15 min)

↓

Washed in PBS once (5 min)

↓

1-3 drops avidin blocking agent added (15 min)

↓

Rinsed in PBS

↓

1-3 drops biotin blocking agent added (15 min)

↓

Rinsed in PBS

↓

Primary antibody added & incubated for overnight at 2-8^{0}C

↓

Washed thrice in PBS (5 mins)

↓

Incubated with 1-3 drops of biotinylated secondary antibody (1 hour)

↓

Washed thrice in PBS (15 min)

↓

Incubated with 1-3 drops of HSS- HRP (30 mins)

↓

Washed thrice in PBS (2 min)

↓

DAB was added and incubated (15 min)

↓

Washed with PBS thrice (10 min)

↓

Rinsed in distilled water (5 min)

↓

Stained with haematoxylin (30 seconds)

↓

Washed in distilled water

↓

Placed in 70% isopropanol (5 min)

↓

Placed in 100% isopropanol (5 min)

↓

Placed in xylene (5 min)

↓

Slides were mounted using DPX

↓

Slides were observed under the microscope

STATISTICAL ANALYSIS

Data entry and descriptive analysis was performed using SPSS version 10.0.5. Mean LI and standard deviation was calculated to assess B7-H3 expression.

- The intensity of staining was determined by two examiners and the inter-examiner variation was determined by kappa analysis.
- Independent 't' test was used to compare the two groups, (Healthy and Chronic Periodontitis) with regard to the B7-H3 expression.

RAGAS DENTAL COLLEGE AND HOSPITALS, CHENNAI.

DEPARTMENT OF PERIODONTICS

PROFORMA

NAME: AGE: SEX: DATE:

ADDRESS: OCCUPATION:

OP.NO:

CHIEF COMPLAINT:

PAST DENTAL HISTORY:

MEDICAL HISTORY:

PERSONAL HABITS:

INTRAORAL EXAMINATION

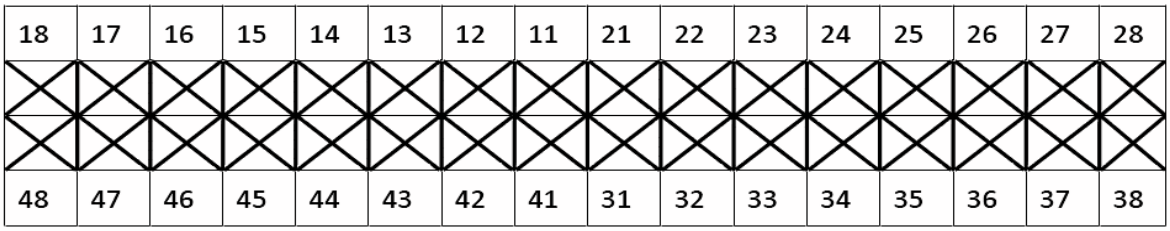
A. HARD TISSUE EXAMINATION:

B. SOFT TISSUE EXAMINATION:

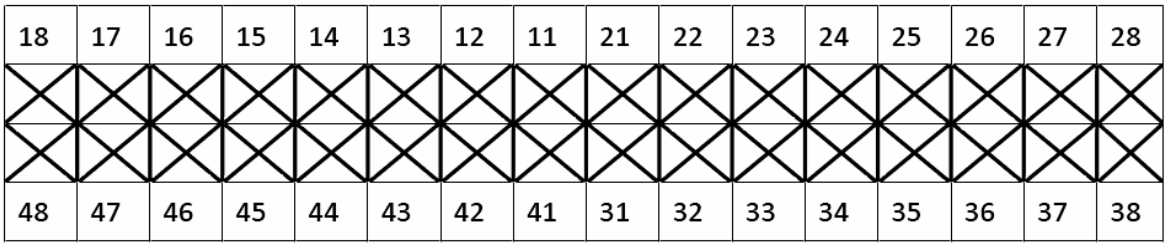
- GINGIVAL FINDINGS

- DENUDED ROOTS (MILLER'S CLASSIFICATION)

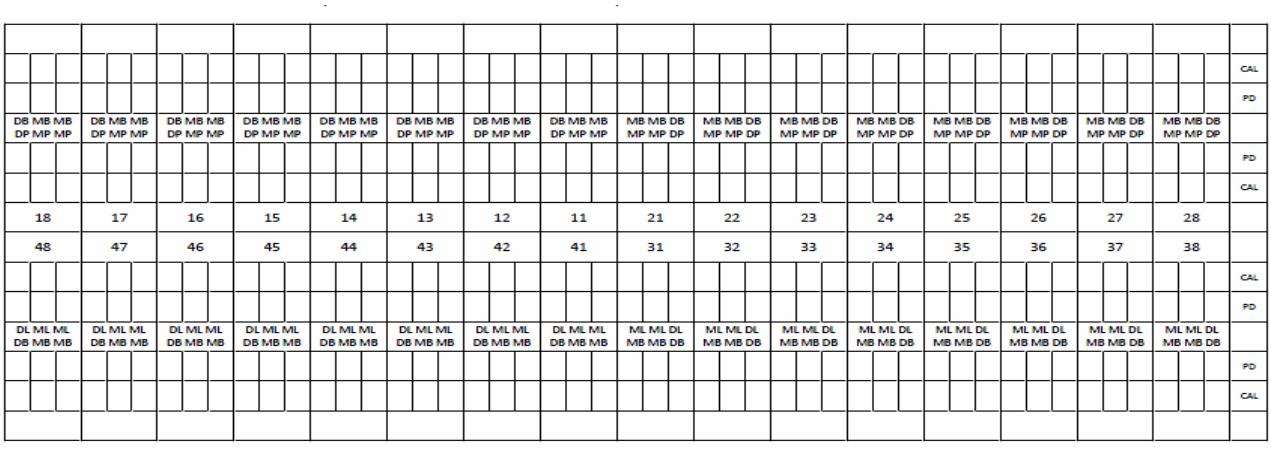
PLAQUE SCORE:



BLEEDING SCORE:



Measurement of Probing Depth and Clinical Attachment Loss



PROVISIONAL DIAGNOSIS:

TREATMENT PLAN:

BIOPSY DETAILS:

CONSENT FORM

I,_____s|o,w|o,d|o_____

_____aged about_____years Hindu/ Christian/Muslim/_____

residing at _____ do hereby solemnly and state as follows.

I am the deponent herein; as such I am aware of the facts stated here under.

I state that I came to Ragas Dental College Hospital, Chennai for my treatment for ……

I was examined by Dr. _____ and I was requested to do the following tests.

1.

2.

3.

I was also informed and explained about the pros and cons of the treatment / test in the _____ (language) known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequences of the dissertation/ thesis /study and I undertake to cooperate with the doctor for the study.

I also assure that I shall come for each and every sitting without fail.

I also authorize the doctor to proceed with further treatment or any other/suitable/alternative method for the study.

I have given voluntary consent to undergo treatment without any individual pressure.

I am also aware that I am free to withdraw the consent at any time during the study in writing.

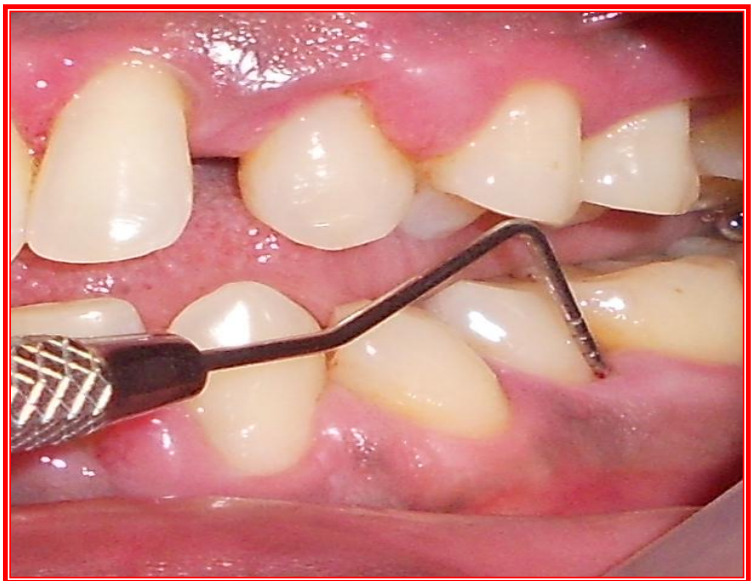
Signature of the Patient/Attendant

The patient was explained the procedure by me and he has understood the same and with full consent signed in (English/ Tamil/ Hindi/ Telugu/ _____) before me.

Signature of the Doctor

HEALTH GROUP (GROUP A)

DISEASE GROUP (GROUP B)



ARMAMENTARIUM

REAGENTS


ACETONE
APES
PBS
1% AMMONIA


Xylene I
Xylene II
Alcohol I 100%
Alcohol II 70%
3% H_2O_2

PRIMARY ANTIBODY

SECONDARY ANTIBODY KIT

RESULTS

A total of 30 gingival tissue samples (15 health and 15 diseased) were evaluated for expression of B7-H3. (The results are summarized in Table 1 and 2).

Staining in epithelium

All the tissues were assessed for intensity of staining in gingival epithelium. Intense staining was observed in 5 specimens in group A and 4 specimens in group B. Moderate staining was observed in 7 specimens in group A and 8 specimens in group B. Pale staining was seen in 3 specimens in group A and 1 specimen in group B. Brown staining was not seen in 2 specimens in group B. Therefore only 13 specimens in group B expressed B7-H3 in epithelium while all 15 specimens in group A were positive for B7-H3 in gingival epithelium.

A good inter-examiner agreement was observed between the two examiners (health - 0.718; disease - 0.797).

Staining in connective tissue

Immunostaining of gingival connective tissue showed localization of B7-H3 to just beneath the basement membrane. The number of positive cells in lamina propria was evaluated using mean labeling index. The results of the mean labeling index were as follows: in group A, the mean labeling index was

50.28±26.09; in group B, 41.37±25.29. There was no statistically significant difference in the mean labeling index between the two study groups, since p value is >0.01 (p value = 0.415).

Figure 1,2,3 and 4 describes B7-H3 expression in health and disease, when viewed under light microscope at 10x and 40x.

Table 1.

Tabulation of Intensity and Labeling Index in Gingival Samples

S. NO.	AGE/ SEX	SITE	PPD	CAL	Procedure	Intensity 1	Intensity 2	LI	SLIDE NO.
1.	21/F	13–P*	2 2 2	-	CL*	++	++	20	4555
2.	49/F	36-B*	3 3 3	-	CL	++	+	75.3	4521
3.	20/F	17-B*	2 3 3	-	CL	+++	+++	-	4519
4.	44/M	36-L*	3 3 3	-	CL	++	+	22.2	4509
5.	32/F	21-P	2 3 3	-	CL	+++	+++	73.5	4627
6.	24/M	16- B	2 1 2	-	CL	++	++	75.3	4342
7.	24/F	37-L	2 3 3	-	CL	+++	+++	69.3	4464
8.	22/M	46-L	3 2 3	-	CL	++	++	-	4437
9.	39/M	27-B	2 2 2	-	CL	+++	+++	71.9	4585
10.	25/F	25-P	2 2 2	-	CL	+	+	13.2	4694
11.	21/F	46-B	3 2 3	-	CL	+++	+++	18.4	4239
12.	26/M	25-B	3 3 3	-	CL	+	+	-	4333
13.	25/F	25-B	3 2 3	-	CL	++	++	-	4592
14.	24/F	36-L	3 3 3	-	CL	++	++	56.1	4234
15.	22/F	13-P	2 2 2	-	CL	+	+	57.9	4345
16.	43/M	27-B	8 6 8	9 8 9	MWF*	++	+	92.6	4674
17.	60/F	11-P	6 6 6	7 7 7	MWF	+++	+++	-	4359
18.	38/F	45-L	6 5 6	7 7 7	MWF	+	+	53.3	4612
19.	48/M	47-B	10 8 8	10 9 9	MWF	++	++	44.7	4488
20.	39/M	46- B	10 6 8	12 7 8	MWF	++	++	26.1	4327
21.	28/F	36-B	4 2 10	6 4 11	MWF	+++	+++	29.8	4683
22.	45/M	25-B	5 7 7	7 8 8	MWF	+++	+++	18.9	4720
23.	55/M	23-B	6 8 8	7 10 9	MWF	++	++	14.3	4719
24.	36/F	17-P	7 6 8	8 7 9	MWF	++	++	49.4	4443
25.	40/F	46-L	8 6 8	9 8 9	MWF	++	++	31.4	4554
26.	58/M	16 –B	7 10 8	9 10 9	MWF	-	-	-ve*	4711
27.	37/F	46-L	6 4 6	7 5 7	MWF	-	+	-ve	4344
28.	37/F	16- B	8 8 8	10 9 9	MWF	+++	+++	26.3	4247
29.	43/M	16- P	9 9 7	10 11 8	MWF	++	++	85.7	4499
30.	48/F	34-B	9 7 9	10 9 10	MWF	++	++	24.0	4540

*B- Buccal, L- Lingual, P- Palatal, CL- Crown Lengthening, MWF- modified Widman flap, -ve- Negative

Table 2.

Comparison of Intensity Scoring and Mean Labeling Index between Health and Disease

	Group A (health)	**Group B (periodontal disease)**	**p value**
Epithelium (intensity)	**Intense - 5** **Moderate - 7** **Mild – 3**	**Intense – 4** **Moderate – 8** **Mild – 1** **Negative - 2**	**-**
Connective tissue (mean LI± SD)	**50.28± 26.09**	**41.37±25.29**	**0.415**

(p value <0.001-statistically significant; p value >0.001- statistically insignificant)

FIG.1: EXPRESSION OF B7-H3 IN HEALTHY GINGIVA (10x VIEW)

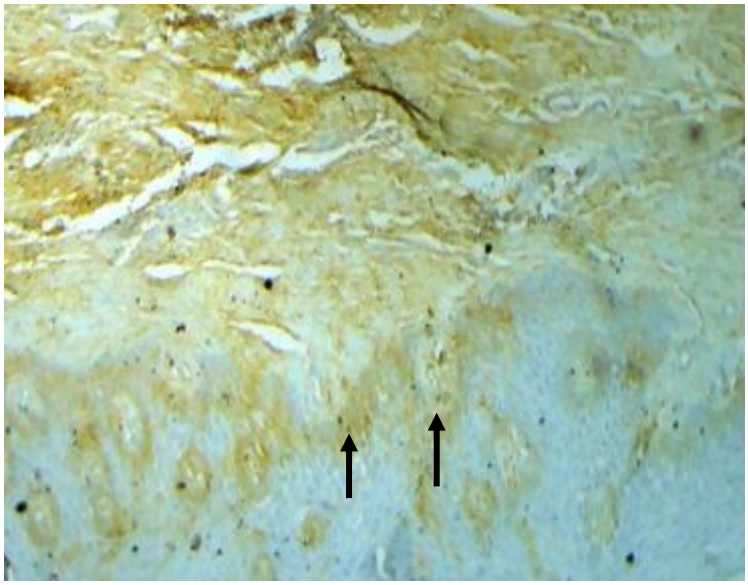
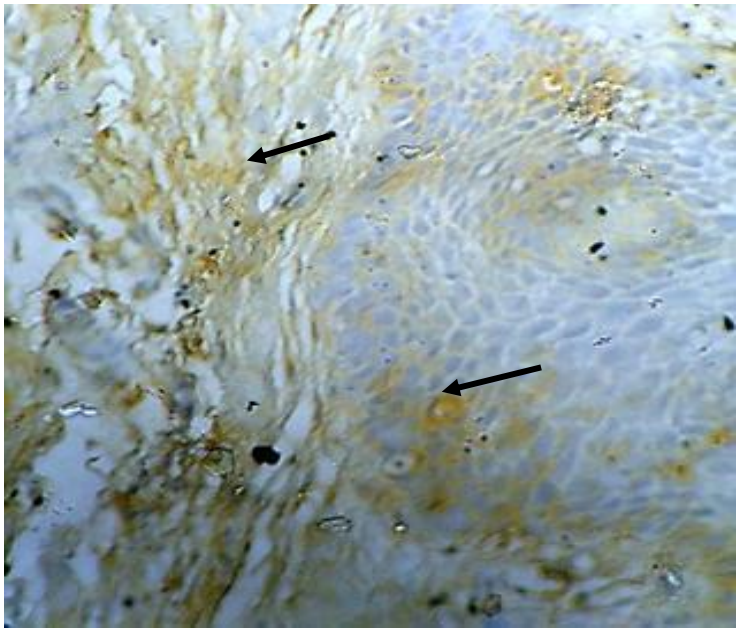


FIG.2: EXPRESSION OF B7-H3 IN HEALTHY GINGIVA (40x VIEW)



↑ - indicates positive staining

FIG.3: EXPRESSION OF B7-H3 IN DISEASED GINGIVA (10x VIEW)

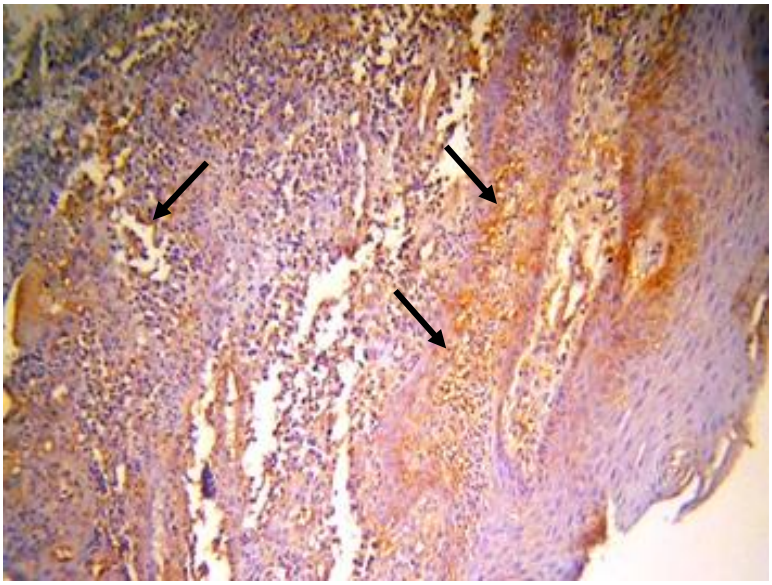
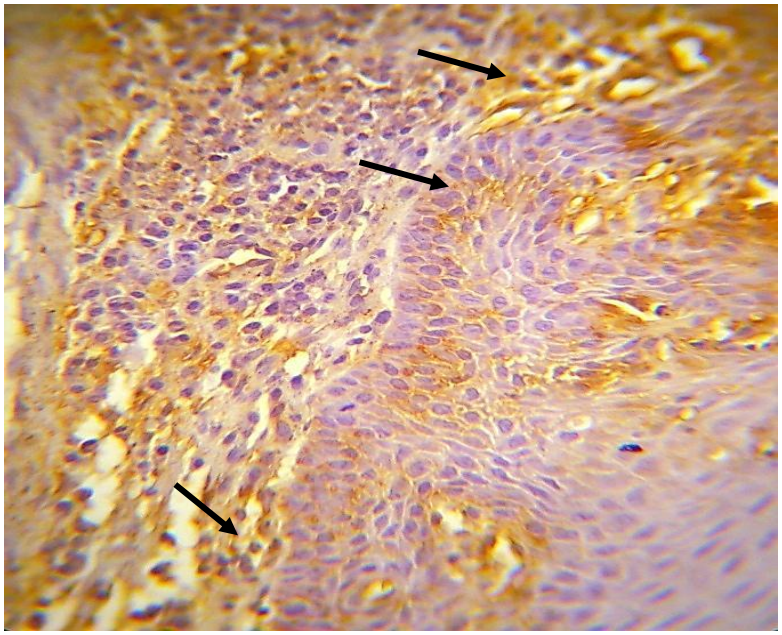


FIG.4: EXPRESSION OF B7-H3 IN DISEASED GINGIVA (40x VIEW)



↑- indicates positive staining

DISCUSSION

Progression of periodontal disease is episodic in nature characterized by short bursts of disease activity followed by a long quiescent phase.[53] This pattern of disease progression is thought to be influenced by the host response, triggered by periodontopathogenic bacteria.

The role of T cells in regulating immune response is well documented. They are crucial for progression and control of chronic inflammation,[100,161] and have been implicated in the pathogenesis of periodontal disease. Optimal T cell function requires a set of stimulatory and inhibitory signal in addition to antigen presentation.

Earlier studies by Gemmell E et al (2002)[49] on B7 molecule expression were based on the premise that, costimulatory molecule are primarily expressed on professional APC leading to activation of T cells. Present evidence suggests that non-immune cells may also express costimulatory molecules. Previous studies in mouse models and humans[23,147,73,138,156,162,29] have identified B7-H3 as a newly discovered co-stimulatory molecule of B7 family. It is an important regulator of T cell function and their results provided the basis for our present study.

Several authors have reported B7-H3 expression to be induced in immune cells such as B cells, monocytes, dendritic cells and T cells[134,23,136,138,165] and by nonimmune cells such as fibroblast-like

synoviocytes and epithelial cells.[61] Though, there exists contradictory evidence for the role of B7-H3 in T cell function, recent evidence suggests an inhibitory role.[19,135,147,73,83]

The present study was undertaken to clarify if there is any quantifiable difference in expression of B7-H3 in healthy and diseased gingival specimens. To our knowledge this is the first study to assess the expression of B7-H3 in gingival tissues. The study comprised of 30 patients (15 health, 15 disease) who were randomly selected from those attending the outpatient clinic of Ragas dental college and divided into two groups as Group A (healthy periodontal tissue as characterised by PD< 3mm and BOP<10% of sites) and Group B (diseased periodontal tissue as characterized by PD ≥5mm and CAL≥ 3mm).

In group A, gingival tissue was excised during crown lengthening procedures while in group B it was removed one week after scaling and root planing, while carrying out the modified Widman flap procedure. Biopsy specimens obtained were fixed in 10% formalin and paraffinised within 48 hours. Antigen retrieval was carried out using EDTA buffer (pH8). Staining was carried out under standardized temperature and time. The slides were viewed under light microscope at magnification 10x and 40x. Presence of brown membrane staining on the cells was taken as positive. The staining was evaluated based on intensity in the epithelium and labeling index in connective tissue.

B7-H3 molecule was examined as:

- cell surface marker
- speculated to play an active role in regulation of T cell activation as previously reported.[23,83,90,116,134, 135,147]

Results of the present study revealed that B7-H3 is expressed in gingival tissue. In the epithelium positive staining was confined to the basal layer. The intensity of staining ranged from intense to moderate and there was no difference in intensity of staining between the groups. In the connective tissue, the staining was observed close to the basement membrane. Our results demonstrated, in the connective tissue a statistically insignificant decrease (p value = 0.415) in diseased specimens (41.37±25.29) when compared to healthy specimens (50.28±26.09).

The results of the present study could be speculated as follows:

The gingiva provides the first line of defense in protection against invading pathogens.[149,25,120] In health, B7-H3 expression may provide signals for increased basal inflammatory tone in response to commensal bacterial antigen. The need for this basal inflammatory tone arises from the fact that putative pathogens may also be present in health, which has to be eliminated.

In disease the decreased expression of B7-H3, could be due to B7-H3 being used up during regulation of T cell function. Conversely it can also be

argued that continous antigenic stimulation may lead to downregulation of peripheral tolerance mechanisms.

Earlier studies have revealed the expression of other members of B7 family. Increased expression of B7-1 and B7-2 has been reported in diseased periodontal specimens.[110,46,26,96] Figueira et al (2009)[40] showed that blockade of PD-1 resulted in upregulation of IFN-γ by T cells in periodontal disease.

Our study is in agreement with studies carried out in gastric carcinoma[156,162] and pancreatic cancer[85] which reveal a decreased expression of B7-H3 in disease. These studies correlate elevated B7-H3 expression with an increased survival rate in patients.

B7-H3 has been found to play a contrasting role in other chronic inflammatory diseases. Tran et al (2008)[147] examined synovial tissue from RA patients. They found maximum B7-H3 expression in lining layer of RA synovium. Suh et al (2003)[135] in murine studies using B7-H3 knockout mice revealed increasing severity of airway inflammation and increased T cell infiltration. Kim J et al (2005)[73] examined B7-H3 expression in nasal epithelial cells of patients diagnosed with asthma and chronic rhinosinusitis. They reported increased expression of B7-H3 with increasing severity of disease. Healthy and chronic rhinosinusitis tissue samples revealed negative or mild staining while intense staining was found in tissue from patients diagnosed with asthma. Expression of B7-H3 has been evaluated by a number

of authors in prostrate cancer.[162,122] They have found that increasing expression of B7-H3 in the specimens correlates with an increased severity of disease and probability of recurrence.

At present factors determining disease progression are not well characterized. Presence or absence of tolerance has been implicated in disease progression. Our results indicate B7-H3 does play a role in pathogenesis of periodontal disease.

The clinical implication based on the results of our study is that, increased expression of B7-H3 may be protective, as it prevents excessive inflammatory response. Hence it can be used in host modulation therapies to regulate T cells function.

Limitations of this study include that antibodies only against B7-H3 molecule were used, as a result does not reveal the cells which express this molecule. The small sample size of this study could also be a reason for the statistically insignificant decrease in B7-H3 expression in diseased specimens.

SUMMARY AND CONCLUSION

This study was carried out to evaluate the expression of B7-H3 in healthy and diseased gingival tissue samples.

15 healthy and 15 diseased gingival biopsy specimens were obtained at random from patients attending the outpatient clinic in Ragas Dental College. These specimens were paraffinised and processed. The thin sections obtained were subjected to immunostaining and slides were viewed under light microscope to evaluate brown staining, indicative of B7-H3 expression. In epithelium intensity of staining was examined while number of positive cells was counted in connective tissue.

Staining in epithelium was found in basal layer, ranged from moderate to intense with no significant variation between healthy and diseased specimens. It was evaluated by 2 examiners to remove bias. Kappa analysis revealed good inter-examiner agreement (health - 0.718; disease - 0.797). In the connective tissue staining was found adjacent to basement membrane with a statistically insignificant ($p = 0.415$) decrease in positive cells from health (50.28 ± 26.09) to disease (41.37 ± 25.29).

This study demonstrates decreased expression of B7-H3 in diseased as compared to healthy gingival samples. This difference between the two groups was statistically insignificant. More number of cases with

statistically significant data is essential to prove its use in host modulation therapy and also to regulate T cell function as a whole.

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